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Dysregulation of the human Transforming acidic coiled coil (TACC) genes is thought to be important in the development of breast cancer. However, the mechanism by which they function still remains to be clarified. We have demonstrated that the full length TACC2 protein can inhibit the tumorigenic phenotype of certain breast cancer cells. We have now performed mapping experiments that demonstrate that the conserved coiled coil domain is critical for this effect. We have confirmed that the histone acetyltransferases (HATs), hGCN5, and pCAF bind to this domain, suggesting that interaction between TACC2 and these proteins may be critical for the maintenance of the normal mammary epithelium. Although breast cancer cells do not significantly express hGCN5, they do express pCAF. A biological function of TACC2 was indicated by its effect to efficiently negate the *in vitro* suppression of DNA-dependent protein kinase (DNA-PK) mediated pCAF activity. Taken together, these findings appear to imply that one of the functions of TACC2 is to counteract a negative modulator of histone acetylase activity, thereby potentially regulating the expression of genes involved in the maintenance of normal mammary development

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INTRODUCTION

The human transforming acidic coiled-coil (TACC) family of genes map to chromosomal regions associated with the development and progression of cancer [1,2]. TACC2 is normally expressed at low levels in normal breast cells [3]. Recently, Chen et al demonstrated that TACC2 mRNA was downregulated in the more malignant clones of the HMT-3522 cell line based model for breast tumor progression [3]. In our first year report, we described the reevaluation of the potential role of TACC2 as a breast tumor suppressor gene, based on our identification of the two major isoforms of TACC2 expressed during development, including the splice variants expressed in the mammary gland. The analysis of TACC2 overexpressing cell lines revealed cell type specific effects on the ability of breast cancer cells to exhibit anchorage independent growth and to migrate through a basement membrane like matrix, suggests that the effect of overexpression of TACC2 on the ability of breast cancer cells to divide in culture could be differentially affected by the genetic background of the original tumor, and may link the function of TACC2 to estrogen signaling.

There is significant evidence to indicate that TACC2, and TACC proteins, in general, are distributed both in the cytoplasm and the nucleus [4], with some of the nuclear localised TACC protein concentrated in nuclear speckles [3]. This indicates that both the TACC proteins and histone acetyltransferases can be physically located in the same subcellular compartment. We have previously determined that TACC2 interacts with the histone acetyltransferase hGCN5. As hGCN5 is a key component of complexes that regulate transcription by acetylating histones and transcription factors [5,6], this suggested that TACC2 could play a role in the regulation of transcription through interaction with this molecule. We demonstrated that, in the human embryonic kidney cell line 293, the native TACC2s isoform co-immunoprecipitates with the histone acetyltransferases hGCN5, pCAF, CBP and p300. As outlined below, we are further characterizing the normal functional role of TACC2, with particular relevance to these interactions with the histone acetyltransferases. This analysis is already providing insights into the role of TACC2 in the normal growth and differentiation of cells, and possible mechanisms by which inactivation could promote tumor development.

This is the second year report for this grant covering the twelve month period from July 1/2002 – Jun 30/2003. At the time of the submission of the previous annual report, the work outlined was under review for publication in Genomics. We were requested to perform some additional experiments, before acceptance of the manuscript. The manuscript was finally published in Genomics, Volume 81 (see Appendix)

BODY

Specific aim 1. Analysis of the effect of full length TACC2 and deletion mutants on growth suppression of breast cancer cell lines.

The finding by Chen et al [3] that the carboxy terminal 571 amino acids of the TACC2 protein could partially reduce the malignant phenotype of one breast cancer cell line suggested that the carboxy terminal TACC domain could be a major determinant of the suppressive effects of TACC2. We have generated three N-terminal deletions and one carboxy-terminal deletion mutants in the mammalian expression vectors pcDNA3, and EGFPC2. In using these constructs, we encountered the predicted problem that low transfection efficiency into breast cancer cells may hamper our interpretation of the results. However, we were able to isolate MDA-MB-468 clones expressing the C-terminal deletion

construct, missing the TACC domain. Unlike the full length TACC2s construct, stable transfectants carrying the C-terminal deletion showed no significant alteration in their ability to form colonies in soft agar and were able to invade and migrate through the Matrigel matrix at levels similar to vector controls. For the N-terminal deletion constructs, no transfectants stably expressing the TACC2 truncation proteins were obtained. To overcome the problem of low transfection efficiencies, we have cloned the deletion constructs into the retroviral vector pLPLNCX. The pLPLNCX vector has the facility to produce a self inactivating retrovirus that can then be used to directly infect target cells at higher efficiency than by transfection of plasmid DNA alone, making this vehicle ideal for introducting our constructs into the breast cancer cell lines. We have now performed infections with the pLPLNCX-TACC2 retroviruses. We have infected 8 cell lines: MCF10A, MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-453, MDA-MB-468, Sk-Br3, T47D (and control mouse fibroblasts). Introduction of the coiled coil into any of the cell lines was cell lethal, and we were unable to identify any clones expressing this domain. However, we were able to generate a small number of clones expressing the region from the SDP repeat motif to the end of the coiled coil (SDP-CC) in MCF10A and the murine fibroblasts, and this was higher than the the number obtained from the full length TACC2. In no case could we obtain TACC2 expressing clones from the other cell lines. This indicates that the coiled coil domain is the major determinant of the TACC2 inhibitory effect, but that this may be ameliorated, in part by the inclusion of the SDP repeat domain, which binds to the GAS41 accessory component of the SWI/SNF chromatin remodeling complex [7]. The difference between the full length construct and the SDP-CC construct might also suggest that the region N-terminal to the SDP repeat may also contain a novel interaction motif.

The mapping of potential modulatory domains of TACC2 in the breast cancer cell lines largely completes the goals outlined in Task 2 and completes Specific Aim 1.

Specific Aim 2: Examination of the role of hGCN5 and the TACC2-hGCN5 interaction in breast tumorigenesis.

We have shown that hGCN5 was expressed at relatively low levels in breast cancer cell lines, although, the closely related gene, pCAF is expressed in the same lines and may therefore represent an alternative target for potential TACC2 mediated repression events. Further analysis of the role of hGCN5 has centered upon the reintroduction of hGCN5 into breast cancer cells. We have cloned the hGCN5 open reading frame, as well as a series of carboxy-terminal deletion mutants lacking the bromodomain, the bromodomain and the ADA2 interaction domain and a C-terminal mutant deleting the histone acetyltransferase domain to the end of the native protein, into pcDNA3, and the EGFPC2 vector. In using these constructs, we have experienced the same problems as detailed above, i.e. low transfection efficiency, and the inability to identify transfectants stably expressing hGCN5 or its deletion constructs. To overcome this problem, therefore, we have cloned hGCN5 into the retroviral vector pLPLNCX. Cloning of the other deletion constructs into these vectors is expected to be completed soon. We have used the pLPLNCX-hGCN5 retrovirus to infect the same cell lines, indicated above, and selected for integration of the retroviral construct. We detected relatively little expression of the hGCN5 construct in the stable clones produced, suggesting selection against hGCN5 expressing clones. In combination with the previous observation that lack of expression of hGCN5 in breast cancer cells, this data further indicates that hGCN5 may be a breast tumor suppressor gene in its own right. This completes Task 4 in the Statement of Work.

To examine the potentially significance of pCAF as an alternative target for TACC2 mediated repression events in breast cancer, we have performed co-immunoprecipitation analysis using an antibody specific for native pCAF. Consistent with our prediction, native TACC2s (and TACC3) was found in the complex containing endogenous pCAF (Fig. 1).

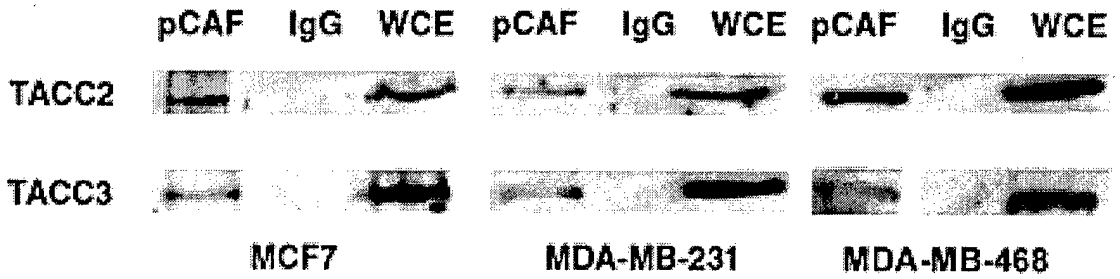

Fig. 1. Immunoprecipitation of TACC2 and TACC3 by pCAF in three breast cancer cell lines.

With the technical difficulties experienced in the analysis of hGCN5 in breast cancer cell lines, and with the commercial availability of newly developed resources to examine the pCAF protein *in vitro*, we have performed *in vitro* analysis of the effect of TACC2 on the histone acetyltransferase (HAT) activity of pCAF (instead of hGCN5, as detailed in Task 6 of the Statement of Work). Several proteins have been shown to bind directly to the region encompassing the HAT and ADA2 interaction domains of pCAF and hGCN5 For example, both adenovirus E1A protein and the human Twist protein directly bind to the HAT domain of pCAF, and p300, thereby downregulating their HAT activities [8,9]. We have now found that TACC2s binds to the HAT domain as well as the ADA2 interaction domain of hGCN5. Because, similar to Twist and E1A, the TACC 2 interact with the region encompasseing the HAT catalytic site, a possibility exists that TACC2s could directly inhibit the HAT activity of pCAF and hGCN5L2. To investigate this, we analyzed the effect of TACC2s on the activity of *in vitro* synthesised pCAF. Results obtained show that TACC2s has no direct effect on the acetylation of histone H4 up to a 12 molar excess of TACC2s to pCAF (Figure 2A), suggesting that, at least for this HAT member, TACC2s is neither a direct accessory component nor a competitive inhibitor which causes steric hindrance at the HAT catalytic site.

To gain clues on a potential biological function of TACC2s, we have begun to test its effect on other factors that may influence HAT regulation. One of the more-characterized mechanisms is the sequestration, serine/threonine phosphorylation and subsequent downregulation of hGCN5 by the DNA-dependent protein kinase (DNA-PK) complex [10], which is mediated by the Ku70 subunit binding to the C-terminal bromodomain [10]. Using an adaption of the *in vitro* assay system previously used by Barlev et al (1998), we found that activated DNA-PK reduces the histone acetylase activity of pCAF by approximately 25% ($p<0.05$) (Figure 2B). This indicated that, similar to hGCN5L2, DNA-PK can negatively regulate pCAF HAT activity. It should be pointed out that these inhibition levels are less than that seen for hGCN5L2 [10]. This may in part be accounted for by subtle differences in the tertiary structure of the proteins themselves, and/or the differences in assay systems used. Under these assay conditions, we found that introduction of exogenous TACC2s can negate this effect and completely restore the pCAF HAT activity (Figure 2B). Thus, although TACC2 binding to pCAF may cause steric hindrance or conformational changes in pCAF, which prevent DNA-PK from phosphorylating the HAT domain, this does not prevent the physical interaction of pCAF with its histone substrate.

Task 6 initially included the effect of TACC2 on the phosphorylation status and HAT activity of native hGCN5. However, the low levels of expression of hGCN5, and the inability to generate hGCN5 expressing stable constructs has hampered the fulfilment of this Task. However, we propose that the finding that TACC2 does not directly alter the HAT activity of a possible alternative target, pCAF, and can inhibit the modulatory effect of Ku70/DNA-PK, at least provides some evidence for a biological function of TACC2 in breast cancer, and is in general compliance with the aims of this task. These findings are currently under review for publication in the journal Oncogene.

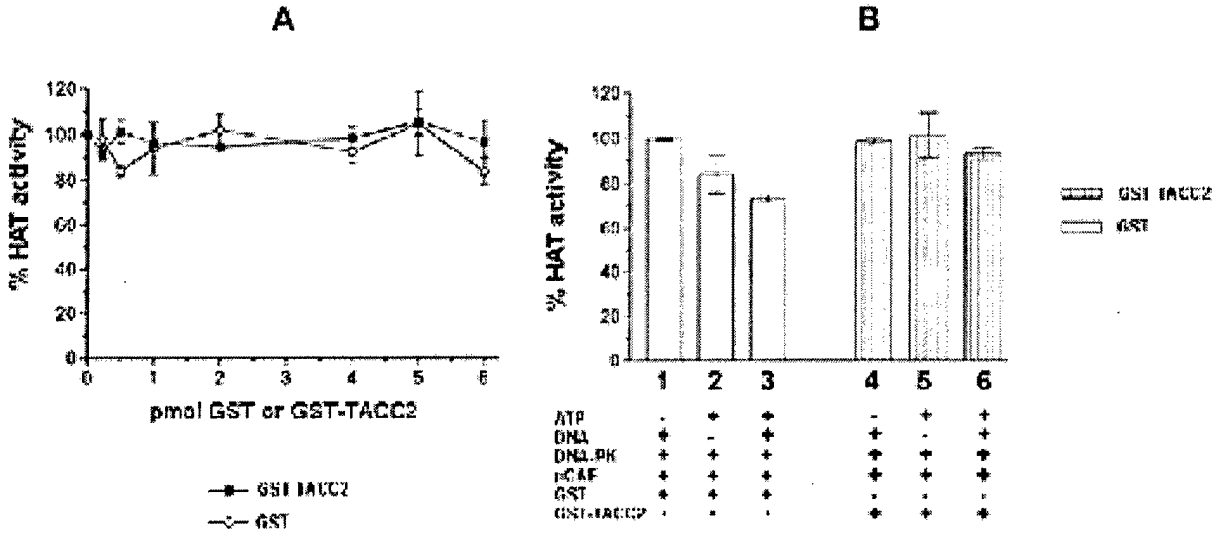

Figure 2. *In vitro* action of TACC2 on histone acetylation by pCAF
(a) TACC2s does not inhibit pCAF HAT activity. HAT assays were performed with 0.5pmol of full length active pCAF in the presence of GST or GST-TACC2 (as indicated). Assay points were performed in triplicate and 100% HAT activity defined as the activity of pCAF in the absence of GST test protein. The activity of the test samples was calculated as a percentage of this value. **(b)** Presence of TACC2s prevents DNA-PK mediated reduction of pCAF HAT activity. 100% activity is the acetylation of histone H4 by pCAF without activation of the DNA-PK (with GST, lane 1; with GST-TACC2, lane 4). The 25% reduction in activity is statistically significant ($P<0.05$) between inactive DNA-PK (Lane 1) and the fully activated DNA-PK (Lane 3). Lack of DNA-PK mediated inhibition of pCAF HAT activity is observed when TACC2s is included in the assay (Lane 6). Data points correspond to the mean of three experiments ±SEM. Activities of the test samples were calculated as a percentage of the respective control.

Specific Aim 3. Characterization of the effect of TACC2 on the transcriptional enhancement of BRCA1 by CBP/p300.

We have demonstrated that TACC2 is found in a complex with CBP/p300 in the HEK293 cell line. Thus, we have proposed that TACC2 and hGCN5 may be present in the complex of CBP/p300 and BRCA1. Downregulation of TACC2 or hGCN5 in breast cancer may therefore represent an alternative mechanism of inactivation of BRCA1 function, comparable with that seen in hereditary breast cancer. We are in the process of performing immunoprecipitations to determine the presence or absence of TACC2 in the BRCA1-CBP/p300 complex in breast cancer cells (Task 7). In our original proposal, we

intended to examine the effect of TACC2 on GAL4-BRCA1 mediated induction of a GAL4 responsive expression cassette containing the chloramphenicol acetyl transferase gene. BRCA1 has been shown to enhance the IFN-gamma mediated induction of the cyclin dependent kinase in p21WAF1 [11]. We would propose, subject to approval, a change in the Statement of Work to investigate the direct effect of TACC2 on the BRCA1-mediated regulation of the p21 promoter, instead of the artificial system previously proposed. To this end, we have already isolated and cloned the full length and the C-terminus of BRCA1 into pcDNA3 (replacing the original Task 8). We will next clone the p21 promoter into the pSEAP reporter vector (BD Bioscience Clontech). Activation of the p21 promoter-SEAP reporter should result in the transcription, synthesis and subsequent secretion of SEAP (secreted alkaline phosphatase) into the tissue culture media of responsive cell lines. The SEAP activity is then determined using a luminometer to measure the light produced by the conversion of the chemiluminescent substrate CSPD by the active SEAP enzyme in the media and the kinetics of induction of the response element can be followed over time by measuring the accumulation of SEAP in the culture media. Transfection efficiencies can be determined by cotransfection of a plasmid that constitutively expresses the DsRed fluorescent protein, and either counting fluorescent cells, manually or using a fluorimeter, at each time point that the SEAP activity is measured. Thus, after normalization, the effect of TACC2 on transcriptional activation from the p21 reporter can be determined by measuring the difference in the accumulation of SEAP in the culture medium of cell lines transfected with the full length TACC2 relative to control cells transfected with vector alone.

NEW RESOURCES AVAILABLE TO Dr. Still

Microplate luminometer/fluorimeter purchased using funds from the Roswell Park Alliance Foundation)

PROPOSED REVISION TO STATEMENT OF WORK

Task 7	Detection of TACC2 in the CBP/p300/BRCA1 complex (months 24-28)
Task 8	Cloning of full length BRCA1 and C-terminal deletion mutant, and p21-SEAP reporter (months 24-26)
Task 9	Determination of the role of TACC2 in the BRCA1 mediated regulation of the p21 promoter (months 26-36)

SUMMARY OF STATUS OF TASKS OUTLINED IN THE STATEMENT OF WORK

Task 1	Complete
Task 2	Complete
Task 3	Complete
Task 4	Complete
Task 5	In progress
Task 6	Complete
Task 7	In progress
Task 8	In progress
Task 9	In progress

KEY RESEARCH ACCOMPLISHMENTS

1) The coiled coil domain is the major determinant of TACC2 inhibitory effects in breast cancer
2) hGCN5 inhibits proliferation of breast cancer cells.
3) TACC2 blocks the Ku70/DNA-dependent protein kinase mediated inhibition of pCAF histone acetyltransferase activity

REPORTABLE OUTCOMES

1) Development of breast cancer cell lines expressing the TACC2 deletion mutants

Publications

1) Molecular cloning, genomic structure and interactions of the putative breast tumor suppressor gene, TACC2. B. Lauffart, O. Gangisetty and **I.H. Still**. Genomics 81: 192-201
3) **Still IH**, Lauffart B,and Gangisetty, O (2002). Analysis of the transforming acidic coiled coil 2 (TACC2) in breast cancer. (Abstract, Department of Defense Era of Hope 2002)
4) Gangisetty, O., Sondarva, G., Vettaikkorumakankauv, A., Jaisani, Z., Lauffart, B., and **Still, I.H**. (2003). The transforming acidic coiled coil proteins are components of multiple protein complexes. (Abstract, American Association for Cancer Research 94th Annual Meeting)

CONCLUSIONS

Through the course of the past 24 months, we have analyzed the effect of the novel tumor suppressor gene, TACC2 and one of its binding partner, hGCN5, on the cellular dynamics of breast cancer cell growth. The inhibitory effect of TACC2 appears to be largely mediated by the TACC domain, a motif which is responsible for the binding of TACC2 to the histone acetyltransferases hGCN5 and pCAF. We have determined that the expression of full length hGCN5 is detrimental to breast cancer proliferation. In combination with the previous observation that breast cancer cells do not express significant levels of hGCN5, this data further indicates that hGCN5 may be a breast tumor suppressor gene in its own right. The findings presented here, together with those from previous studies, clearly suggest that TACC proteins may perform an assembly or coordination function bringing elements of the chromatin remodeling, transcriptional and posttranscriptional machinery together in the nucleus. In addition, by demonstrating that TACC2 can efficiently negate the *in vitro* suppression of DNA-dependent kinase mediated pCAF histone acetyltransferase activity, it would appear that one function of the TACC2 protein in these complexes may be to counteract a negative modulator of histone acetylation activity. Thus, even though the exact downstream targets of DNA-PK mediated HAT modifications remains to be defined, it is likely that TACC2 plays a distinct role as a molecular switch, negating DNA-PK inhibition of HAT activity, permitting pCAF/hGCN5 to acetylate histones and potential regulatory targets interacting with the TACC2-HATF complex. Taken together, these findings appear to imply that one of the functions of TACC2 is to counteract a negative modulator of histone acetylase activity, thereby potentially regulating the expression of genes involved in the maintenance of normal mammary development.

REFERENCES

1. Still, I. H., Hamilton, M., Vince, P., Wolfman, A., and Cowell, J. K. (7-8-1999) Cloning of TACC1, an embryonically expressed, potentially transforming coiled coil containing gene, from the 8p11 breast cancer amplicon. Oncogene 18, 4032-4038
2. Still, I. H., Vince, P., and Cowell, J. K. (6-1-1999) The third member of the transforming acidic coiled coil-containing gene family, TACC3, maps in 4p16, close to translocation breakpoints in multiple myeloma, and is upregulated in various cancer cell lines. Genomics 58, 165-170
3. Chen, H. M., Schmeichel, K. L., Mian, I. S., Lelievre, S., Petersen, O. W., and Bissell, M. J. (2000) AZU-1: A Candidate Breast Tumor Suppressor and Biomarker for Tumor Progression. Mol.Biol.Cell 11, 1357-1367
4. Pu, J. J., Li, C., Rodriguez, M., and Banerjee, D. (2001) Cloning and structural characterization of ECTACC, a new member of the Transforming acidic coiled coil (TACC) gene family: cDNA sequence and expression analysis in human microvascular endothelial cells. Cytokine 13, 129-137
5. Ogryzko, V. V., Kotani, T., Zhang, X., Schlitz, R. L., Howard, T., Yang, X. J., Howard, B. H., Qin, J., and Nakatani, Y. (7-10-1998) Histone-like TAFs within the PCAF histone acetylase complex [see comments]. Cell 94, 35-44
6. Martinez, E., Kundu, T. K., Fu, J., and Roeder, R. G. (9-11-1998) A human SPT3-TAFII31-GCN5-L acetylase complex distinct from transcription factor IID [published erratum appears in J Biol Chem 1998 Oct 16;273(42):27755]. J.Biol.Chem. 273, 23781-23785
7. Lauffart, B., Gangisetty, O., and Still, I. H. (2003) Molecular cloning, genomic structure and interactions of the putative breast tumor suppressor TACC2. Genomics 81, 192-201
8. Hamamori, Y., Sartorelli, V., Ogryzko, V., Puri, P. L., Wu, H. Y., Wang, J. Y., Nakatani, Y., and Kedes, L. (2-5-1999) Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. Cell 96, 405-413
9. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (7-25-1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature 382, 319-324
10. Barlev, N. A., Poltoratsky, V., Owen-Hughes, T., Ying, C., Liu, L., Workman, J. L., and Berger, S. L. (1998) Repression of GCN5 histone acetyltransferase activity via bromodomain- mediated binding and phosphorylation by the Ku-DNA-dependent protein kinase complex. Mol.Cell Biol. 18, 1349-1358
11. Ouchi, T., Lee, S. W., Ouchi, M., Aaronson, S. A., and Horvath, C. M. (5-9-2000) Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-gamma target genes. Proc.Natl.Acad.Sci.U.S.A 97, 5208-5213

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Molecular cloning, genomic structure and interactions of the putative breast tumor suppressor TACC2

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Abstract

The human transforming acidic coiled-coil 2 (*TACC2*) gene has been suggested recently to be a putative breast tumor suppressor. Now we can report the cloning of full length TACC2 cDNAs corresponding to the major isoforms expressed during development. The *TACC2* gene is encoded by 23 exons, and spans 255 kb of chromosome 10q26. In breast cancer cell lines, TACC2 is expressed as a 120 kDa protein corresponding to the major transcript expressed in the mammary gland. Although only slight differences in the expression of TACC2 in normal versus breast tumors were observed, overexpression of TACC2 can alter the in vitro cellular dynamics of some breast cancer cell lines. Significantly, we demonstrate that TACC2 interacts with GAS41 and the SWI/SNF chromatin remodeling complex. This suggests that defects in TACC2 expression may affect gene regulation, thus contributing to the pathogenesis of some tumors.

Keywords: TACC; Breast cancer; Chromosome 10; GAS41; INI-1; SWI/SNF

Introduction

The identification of potential oncogenes and tumor suppressor genes is important in the elucidation of the pathways that govern cell growth, division, and differentiation. Recently, we identified a family of evolutionary conserved genes, the *TACC* (transforming acidic coiled coil)-containing family, which are expressed in a temporal and tissue-specific pattern during development [1,2]. The proteins encoded by these genes are highly acidic and contain a conserved 200 amino acid coiled-coil domain, the TACC domain, which is predicted to play an important role in the function of these proteins [1,2].

Growing evidence supports a role for the TACC family in processes underlying the development of cancer. The human *TACC* orthologues map to regions associated with tumorigenesis and progression. *TACC1* and *TACC2* are located in chromosome 8p11 and 10q26, respectively, two regions that are implicated in breast and other tumors [1,2], while *TACC3* maps to 4p16, within 200kb of a translocation breakpoint cluster region associated with multiple myeloma [2]. In vitro and in vivo studies also indicate that the TACC proteins are linked to the processes of cell growth and differentiation. TACC1 and TACC3 are expressed at high levels during embryogenesis and are then downregulated in differentiated tissues, being expressed either at low levels, or only in restricted tissues [1–3], suggesting that they may play a role in the processes that promote cell division before the formation of differentiated tissues. The essential role of one of the *TACC* genes, TACC3, in development has recently been demonstrated, because homozygous knockout mice die during mid to late gestation [4].

Recently, in the HMT-3522 cell line-based model for breast tumor progression, Chen and colleagues [5] demonstrated that TACC2 mRNA is downregulated in the more malignant clones of the series. These authors also reported the cloning of a 3.8 kb TACC2 cDNA (named AZU-1), encoding a protein of 571 amino acids and predicted molecular mass 64 kDa of these malignant cells. Reintroduction of this cDNA into the malignant breast tumor cells reduced their ability to grow and metastasize [5]. Therefore, this suggested that *TACC2* is a breast tumor suppressor gene, the downregulation of which is an important step

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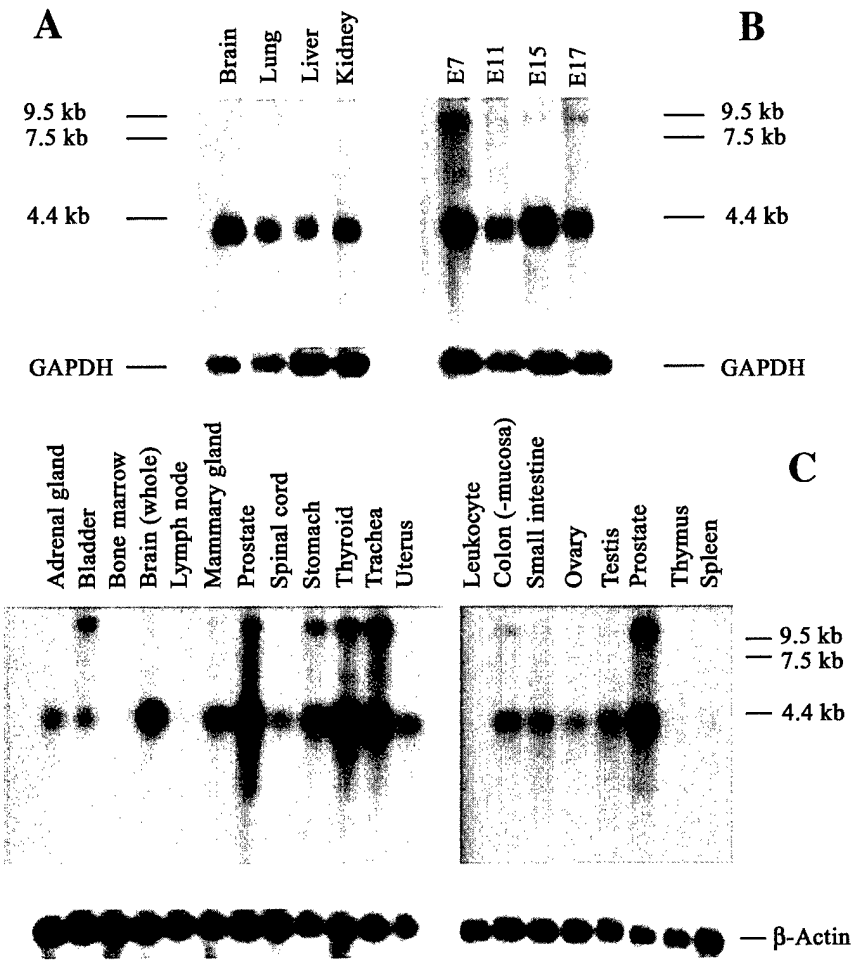

Fig. 1. Northern blot analysis of TACC2. (A) The predominant TACC2 transcript expressed in human fetal tissues is 4.2 kb long. (B) During murine embryogensis, the 4.2 kb transcript is the major isoform. A minor 9.7 kb transcript is also detected. (C) In adult tissues, the most common transcript detected is the 4.2 kb transcript. The 9.7 kb transcript is detected at relatively high levels in trachea. TACC2 is not significantly expressed in tissues of immune and hematological origin.

during breast tumorigenesis. *TACC2* was also recently identified as an erythropoietin-inducible gene in human microvascular endothelial cells [6]. In this case, a 3.3 kb cDNA was described (named ECTACC) that showed significant nucleotide identity with the AZU-1 sequence, but that encoded a larger protein with a predicted molecular mass of 100 kDa.

In this report, we describe the cloning and expression of the *TACC2* gene. We have characterized the major isoforms of TACC2 proteins expressed during development, and identified the splice variant expressed in the mammary gland. We demonstrate using the available genomic and cDNA sequence data that both AZU-1 and ECTACC may contain cloning artifacts, thereby explaining the discrepancies in their sequences. In light of these findings, we have reevaluated the potential role of *TACC2* as a breast tumor suppressor gene. We also show that, similar to TACC1, the TACC2 protein binds to GAS41, and forms a complex with the SWI/SNF core subunit INI-1. This suggests that the suppressive effects of TACC2 may be mediated by interactions with a potential transcription factor complex.

Results

Northern blot analysis of TACC2 in human and mouse tissues

During the cloning of TACC1, we isolated a 1 kb clone, FL4, that was highly related to the 3′ end of the TACC1 open reading frame [1], and thus defined a new gene, which we termed *TACC2* [1]. As a first step toward the characterization of this member of the *TACC* gene family, we performed Northern blot analysis of human and murine tissues using a TACC2-specific probe (Fig. 1). A single 4.2 kb transcript was detected in the human fetal tissues tested (Fig. 1A). This transcript was also detected at all stages of murine embryonic development, in addition to a lower

abundance transcript of approximately 10 kb (Fig. 1B). Both transcripts were evident in a number of adult human tissues, although the relative abundance of each varied depending on the tissue. For example, the 4.2 kb transcript was the predominant isoform in most TACC2- expressing tissues, and is highly expressed in whole brain, as well as prostate, thyroid, and trachea. The larger transcript is expressed at lower levels in a number of tissues, but is a major RNA species in the bladder (Fig. 1C), placenta, skeletal, as well as heart muscle (data not shown). TACC2 does not appear to be expressed at significant levels in the bone marrow, lymph node, or in peripheral blood leukocytes.

Cloning of the transcripts corresponding to the major forms of TACC2

A BLAST search with the FL4 sequence originally identified a single UNIGENE cluster of cDNAs, HS90415, which included approximately 2.4 kb of the 3′ end of the TACC2 transcript. To determine the full sequence of the 4.2 kb major transcript, we used FL4 to screen a fetal brain library to isolate additional TACC2 cDNAs. Sequence analysis of these clones extended the TACC2 sequence and identified an additional human UNIGENE cluster HS202303. A 3,686 bp contig for the human fetal cDNA (GB:AF095791) was assembled, but it could not be extended farther 5′, either through cDNA library screening or 5′RACE. During this analysis, two laboratories independently reported the cloning of cDNAs corresponding to TACC2. The GB:AF220152, named ECTACC, is 3311 bp long with a 5′ untranslated region (UTR) of 369 bp encoding a protein of 906 amino acids [6]. A second TACC2 cDNA, named AZU-1, contained an open reading frame (ORF) of 571 amino acids, with a 5′ UTR of approximately 1.6 kb [5]. However, the ORF of this latter cDNA could be extended 1.3 kb upstream of the proposed start methionine of AZU-1, in agreement with the predicted protein sequences from ECTACC and AF095791. This suggested the possibility that the AZU-1 cDNA represents a RACE cloning artifact, or corresponds to an incomplete splicing product from the other TACC2 isoforms.

We have previously shown that the *TACC2* gene is located on chromosome 10q26, in the same interval as *FGFR2* [1]. Examination of the public human genome database revealed that the available TACC2 sequence was contained within a single BAC clone, RP11-296H2 (GB: AL135793). A comparison of the sequence of this BAC with AF095791 and AF220152 revealed that the two cDNAs diverge upstream of a potential splice acceptor (Fig. 2). Similarly, the AZU-1 nucleotide sequence diverged upstream of another potential splice acceptor. Sequences identical to the 5′ region of AZU-1 are also present in two cDNAs in the UNIGENE database, and BAC RP11-296H2, suggesting that they may correspond to additional exons of TACC2. However, because these putative upstream exons contained stop codons in all three reading frames, and we failed to identify them in rt-PCR analysis from breast, colon, or prostate RNA, it appeared likely that they did not represent the legitimate 5′ end of the TACC2 cDNA.

Analysis of the genomic sequence within 200 kb of the 5′ region of the cloned TACC2 sequence revealed two hypothetical genes, Hs10_27257_29_3_1 and Hs10_27257_29_3_2 with predicted transcript sizes of 5673 nt and 1491 nt, respectively (Fig. 2). When these predicted transcripts were used to search the EST database, several cDNAs were identified that not only linked the two predictions, but also, through cDNA sequences BG822027, BF992557, and BF36966, linked Hs10_27257_29_3_2 to the TACC2 UNIGENE cluster HS272023, which suggested that the TACC2 transcription initiation start is located approximately 150 kb upstream of the 5′ sequence of AF095791 and AZU-1. To confirm that the predictions did correspond to a bona fide TACC2 transcript, we next designed a series of primers for use in rt-PCR. Overlapping PCR products stretching the length of the genescan/TACC2 predictions were amplified from colon, prostate, and mammary gland RNA. Sequence identity of these products was confirmed and the complete sequence generated by this analysis corresponded to a transcript of 9773 bp, which is similar to the length of the largest TACC2 transcript (TACC21) identified by Northern blot analysis. Using primer combinations T2F77/T2R6558 and T2F340/155R, rt-PCR analysis of mammary gland RNA revealed that the smaller 4.2 kb transcript (TACC2s) was produced by the alternative splicing of this product, mainly resulting from the exclusion of a 5313 bp exon (see below). Thus, the main form of TACC2 expressed in the mammary gland is 4146 bp long. The sequences of these two forms have been deposited in GenBank with the accession numbers: AF528098 (TACC21 isoform) and AF528099 (TACC2s isoform).

TACC2 protein structure

The two major transcripts of human *TACC2* are 4.2 kb long and approximately 9.7 kb long. They encode predicted proteins of 1094 amino acids and 2948 amino acids with molecular masses of 119 kDa and 310 kDa, respectively. The size of both TACC2 proteins is significantly larger than that of TACC1 (805 amino acids) and of TACC3 (838 amino acids) [1,2]. Similar to the two other human TACC family members, both TACC2 proteins are highly acidic with a pI of 4.6 and 4.9, respectively, being enriched in acidic (16.2% and 15.3%), proline (10.4% and 10.4%), and serine (11% and 11.2%) residues. Both products contain a number of strong PEST sequences, suggesting that the TACC2 protein(s) may be subject to regulated degradation. This may explain the ladder of multiple smaller forms of the TACC2 protein ranging in size from 120 kDa-60 kDa, originally detected by other groups using Western blot analysis [5,7]. TACC2 also contains several basic regions, which may serve as nuclear localization signals. Indeed, we

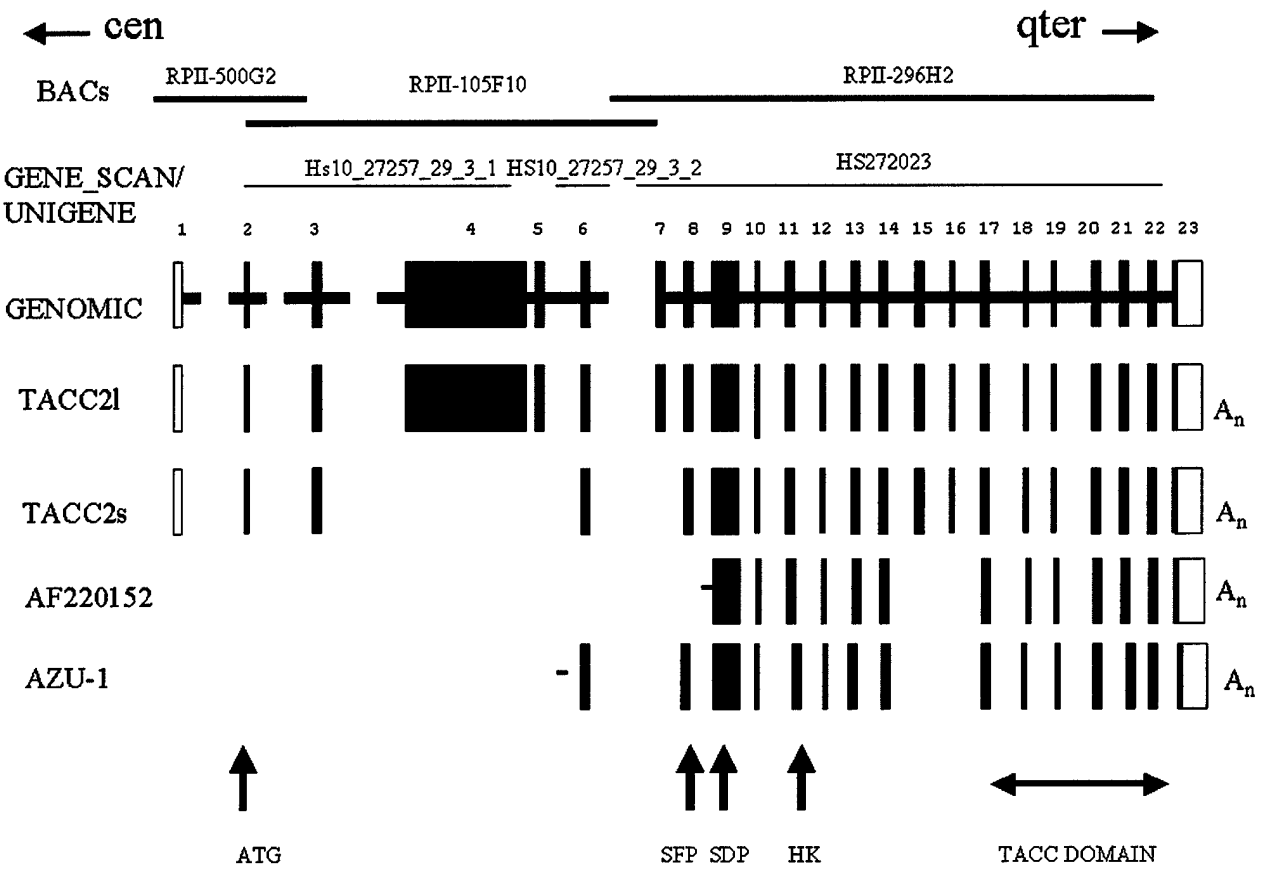

Fig. 2. Genomic structure of *TACC2:* Comparison of the previously cloned TACC2 cDNAs, genome scan predictions, the TACC2 cDNAs reported in this manuscript, and the genomic structure of TACC2. Exons are depicted as black boxes with introns as intervening lines. The SFP motif is located in exon 8. PEST sequences are spread over exon 8 and 9, the two SDP repeats are located in exon 9, and the HATDEEKLA (HK) motif in exon 11. The coiled-coil domain of TACC2 is dispersed over exons 17–23.

have shown that, similar to all the TACC proteins, TACC2 can also be detected in the nucleus, as well as the cytoplasm of interphase cells [7]. Thus, localization of TACC2 to the nucleus may be regulated by the use of these signals.

As previously noted, the TACC2 coiled-coil domain is highly related to the coiled-coil domains of TACC1 (74% identity) [1], and to a lesser extent to TACC3 (53% identity) [2], Xenopus maskin protein (56% identity) [8], and the D-TACC protein (26% identity, 55% similarity) [9]. Apart from the coiled-coil domain, the human TACC proteins share limited regions of homology. Both TACC1 and TACC2 contain a 16 amino acid SFP motif SSDSE-X_2-FETPE-X_2-TP, and an absolutely conserved string of nine amino acids, HATDEEKLA (Fig. 2). Neither of these motifs is found in other TACC proteins, or proteins unrelated to the TACC family, suggesting that they are likely to play a role specific to TACC1 and 2. Additionally, TACC2 contains two copies of the 33 amino acid SDP repeat, which are conserved between the members of the vertebrate TACC family [10]. We have recently demonstrated that the region of TACC1 containing these repeats serves as the binding site for the SWI/SNF component/accessory factor, GAS41 [10].

Genomic organization and multiple splicing of the TACC2 gene

Reconstruction of the TACC2 transcript sequences and comparison with the genomic sequence of chromosome 10q26 revealed that the *TACC2* gene is spread over 23 exons, and is approximately 255 kb long (Table 1). The exons range in size from 61 bp (exon 10) to 5313 bp (exon 4), with introns ranging in size from 247 bp (intron 20) to more than 51 kb (intron 7). The intron/exon boundaries conform to the GT-AG splicing paradigm. The first exon comprises the majority of the 5′ UTR, with the start methionine located in the second exon. The motifs, which are conserved in different TACC proteins, are also located in separate exons: the SFP motif in exon 8, the two SDP repeats in exon 9, and the HATDEEKLA motif in exon 11. The coiled-coil domain of TACC2 is dispersed over the final seven exons, exons 17–23 (Fig. 2).

The major isoforms of TACC2, the 9.7 kb and 4.2 kb transcripts, mainly differ by the inclusion of the unusually large 5.3 kb exon 4. Analysis of the publicly available mouse genome database (contig NW_000333) suggests that an analogous exon is present in the *TACC2* gene on mouse

Table 1
Details of the genomic structure of TACC2. The exon/intron structure, sequence of the intron/exon junctions, and the approximate size of the exons and introns are given

Exon	Exon size (bp)	3′ Acceptor site	5′ Donor site	Intron size (bp)
1	296	N/A	GTGCGgtaa	32446
2	79	tttcttccagTCACC	ACCAGgtgg	28418
3	113	cctggctcagAGGAC	GTCCAGgtag	32091
4	5313	atattttcagCATTG	TGACAGgtac	517
5	114	attatcccagAGAGA	AGAAAGgtca	43659
6	126	ctctccccagTTCAC	CCCAGAGgtac	10871
7	135	gtgtccccagCATCT	CAGCAGgtat	51303
8	137	ctctcatcagGAGTT	AACCAGgtaa	15220
9	1312	ttgtttccagGATGT	AAGGACgtaa	3683
10	61	ttgtttccagTGACA	TCCCAGgtac	1176
11	202	ctctttgcagGACCC	CTGAGGgtaa	7898
12	62	tgttttgcagAGTTG	TTACCTgtaa	1579
13	116	gaccctgcagCAGGA	TTCAGGgtat	1356
14	172	cttcccccagGTCAA	GAAATTgtaa	1338
15	141	cggctttcagACAGC	CTCCAGgttt	865
16	90	ctgtgggcagGAGGA	GCCAAGgtacc	6955
17	144	gattccacagAGAGA	GCAGAGgtatt	423
18	77	ctgaaactagATCAT	AATGAGgtca	3921
19	44	tctcttatagGAAAA	TGATAGgtag	6642
20	161	ctcctgccagAGGAC	CGCAAGgtag	247
21	107	gctgtgtcagAATGA	GGACAGgtaa	388
22	121	gtctccgcagGGCCA	CAGAAGgtaa	4326
23	63bp+3′ UTR	tcacttgcagAATAA	—	—

chromosome 7 (data not shown). Additional alternative splicing is evident in the region preceding the coiled-coil motif. Both AF220152 and AZU-1, as well as associated cDNAs, are missing exons 15 and 16 (Fig. 2). We have performed rt-PCR analysis of this region, detected these shorter transcripts, and a transcript missing only exon 16 in a number of fetal and adult human RNA samples (data not shown). However, the significance of this alternative splicing is unclear, because these exons do not encode a known functional motif.

Expression of the TACC2 protein in breast cancer cells

Commercially available antibodies raised against TACC2-specific peptides have recently become available. The technical specification of antibody 07-228 (Upstate Biotech.) suggests that this TACC2 antibody recognizes a molecular species of 63 kDa in HeLa cells. However, in HEK293 cells (human embryonic kidney), this antibody detects a protein of 120 kDa (Fig. 3), which is in agreement with the predicted protein corresponding to the 4.2 kb transcript expressed in human fetal kidney (Fig. 1A). This isoform was also detected in protein extracts from normal brain, as well as a number of different cell lines (data not shown).

It has been suggested that TACC2 is significantly downregulated in breast cancer cell lines relative to the immortal but not transformed breast epithelial cell line MCF10A [5]. With the observation that the TACC2 protein detected previously [5] may be the result of degradation or aberrant splicing events, we analyzed by Western blotting the total protein extracts from 70% confluent cultures of breast cancer cell lines. TACC2 is predominantly expressed as the 120 kDa isoform in all breast cancer cell lines tested, although no dramatic difference in TACC2 protein levels could be detected at this level of resolution (Fig. 3A). TACC2 mRNA levels are also only slightly reduced in matched breast tumor compared to normal breast tissue pairs when a TACC2- specific probe was hybridized to a commercially available multiple cancer profiling array (BD Biosciences, Clontech, USA) (Fig. 3B), suggesting that only subtle, if any, changes in TACC2 expression may be present in breast tumors.

Assessment of TACC2 as a breast cancer growth suppressor

Previous analysis of the effect of TACC2 on the growth characteristics of breast cancer cells utilized the partial AZU-1 cDNA clone [5]. To determine whether the effects noted previously were due to the use of a partial clone, and therefore may be due to a dominant negative effect, we repeated the in vitro growth experiments of Chen and colleagues [5] in two different human breast cancer cell lines. To investigate the consequences of increased expression levels of TACC2, we introduced a plasmid construct (EGTACC2), which expresses the TACC2s isoform fused to the green fluorescent protein (EGFP) into MCF7 and MDA-MB-468, and stable cell lines were selected, as previously described [1]. The expression of the fusion protein

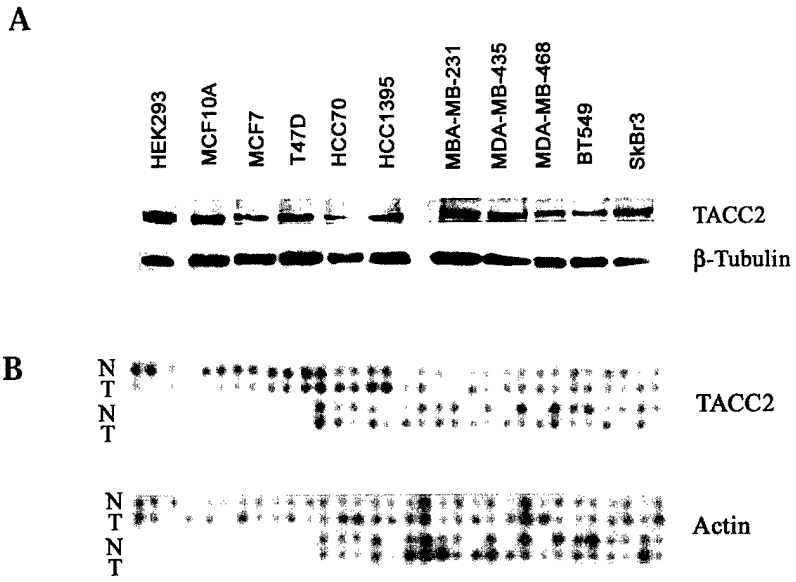

Fig. 3. (A) Western blot analysis of TACC2 protein expression in breast cancer cell lines. TACC2 is expressed as the 120 kDa isoform, corresponding to the predicted open reading frame from the 4.2 kb transcript. 10 μg of protein derived from 70% confluent cultures was loaded in each lane of the Western blot analysis. Even loading was confirmed using a β-tubulin antibody. (B) Analysis of the expression of TACC2 mRNA in breast cancer samples using the Clontech Cancer Profiling Array. This blot contains SMART-amplified cDNA from tumor (T) and matched normal (N) tissues. The blot was also hybridized with actin to confirm even sample loading.

was verified by fluorescence (Fig. 4A), and Western blot analysis demonstrated correct and equivalent expression of the EGFP fusion protein in all cell lines (data not shown). In the cell proliferation assay, TACC2 overexpression reduced the growth rate of the transfected MDA-MB-468, but only slightly inhibited the growth of MCF7 within the assay period (Fig. 4B).

An important indicator of whether a gene product is a potential oncogene or tumor suppressor is its ability to impart, or abolish anchorage independent growth in vitro. Therefore, we determined whether transfection of EGTACC2 into MCF7 and MDA-MB-468 cells would alter cellular motility and growth in soft agar. TACC2 overexpression in three stable transfectants produced no significant alteration in the ability of MCF7 to form colonies in soft agar ($P = 0.11$); however, in MDA-MB-468 the number of TACC2-overexpressing colonies was significantly reduced ($P = 0.01$) when compared to controls (Fig. 4C). MCF7 has previously been shown to migrate poorly through a basement membrane matrix (Matrigel), and transfection of EGTACC2 into MCF7 failed to alter migration rates. However, EGTACC2/MDA-MB-468 transfectants were significantly impaired in their ability to invade and migrate through the Matrigel matrix ($P = 0.001$) within a 24 hour period.

TACC2 complexes with GAS41 and the SWI/SNF core component INI-1

Both large and short isoforms of TACC2 contain two copies of the SDP repeat motif [10]. We have shown recently that the region of TACC1 containing these repeats serves as the binding site for the SWI/SNF component/accessory factor, GAS41 [10]. The degree of conservation of this region between the TACC family members suggests that GAS41 may be a common binding factor for the TACC family [10]. To determine whether TACC2 could also bind this protein, we performed co-immunoprecipitation assays in the EGGAS41/HEK293 cell line that was used for the initial characterization of the GAS41-TACC1 interaction [10]. As shown in Fig. 5, GAS41 is found in a complex with TACC2s in these cells. To confirm further that the interaction between GAS41 and TACC2s is direct, we also performed in vitro GST-pull down assays. Fig. 5B clearly demonstrates that the interaction between GAS41 and TACC2s is direct, and can occur independently of any interaction with TACC1. Furthermore, immunoprecipitates of the SWI/SNF core subunit INI-1 in two breast cancer cell lines, MDA- MB-231 and MCF7, also contain TACC2, thus showing that TACC2 can, at least indirectly, associate with an INI-1 containing SWI/SNF complex (Fig. 5C).

Discussion

We have now reported the cloning, expression, and genomic organization of the *TACC2* gene. TACC2 is expressed as two major isoforms during development; the 4.2 kb transcript is the more abundant form, however, in differentiated tissues the relative levels of the two isoforms can be different, depending on the nature of the tissue. Both isoforms can be expressed in the same tissue, although each transcript can be expressed independent of the other. Thus,

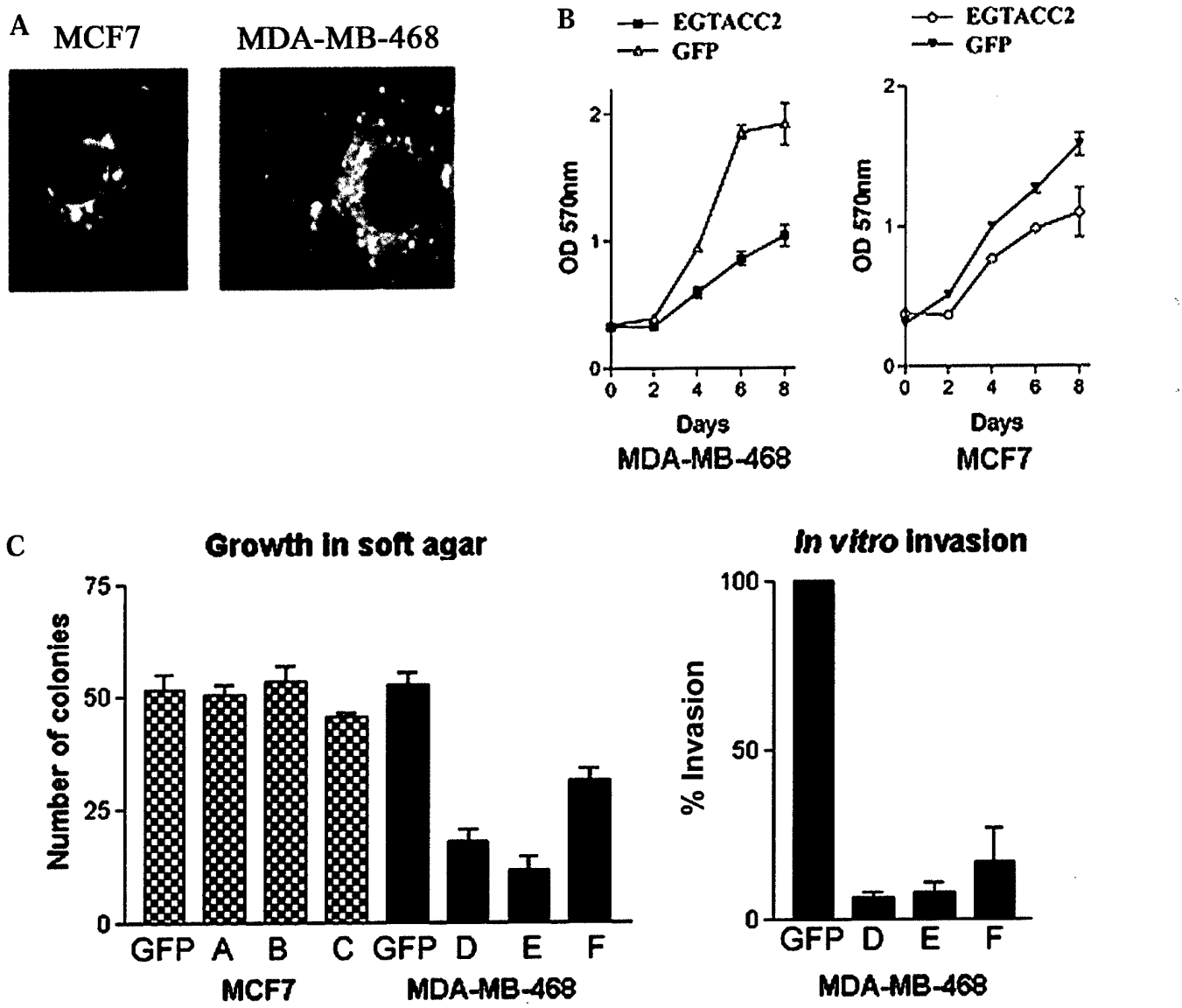

Fig. 4. Analysis of the effect of EGTACC2 expression in breast cancer cell lines. (A) Expression of EGTACC2 in representative live breast cancer cell lines as determined by fluorescence microscopy. Overexpression results in an accumulation of the fusion protein in the cytoplasm, as previously noted in other cell lines [7]. EGFP by comparison was uniformly distributed throughout the cell (data not shown). (B) Cell proliferation curves for EGTACC2 transfected MDA-MB-468 and MCF7. One stable transfectant line of each is shown. (C) The in vitro growth characteristics of TACC2 overexpressing MDA-MB-468 and MCF7 transfectant cell lines were analyzed as described in [5]. No significant difference between the formation of colonies in soft agar was noted for MCF7-EGTACC2 transfectants A–C ($P > 0.05$) compared to the control MCF7 cells expressing EGFP alone. However, both the ability of MDA-MB-468 clones D–F to form colonies in soft agar and migrate through Matrigel was reduced by overexpression of TACC2 ($P < 0.05$ in each case). Data points correspond to the mean of three experiments ±SEM.

the 4.2 kb transcript is the predominant transcript in the mammary gland, but expression of the larger 9.7 kb transcript is increased in tissues that contain a high proportion of either smooth or striated muscle. Similar to the other TACC proteins, both transcripts encode highly acidic proteins, which are rich in acidic, proline, and serine residues. As previously noted [1,5], the TACC2 coiled-coil domain is most similar to TACC1 and, from database analysis, TACC2 has orthologues in mouse, Xenopus, and the pufferfish (Still and Liang, unpublished), suggesting that these genes are the result of a duplication event that occurred after the invertebrate/vertebrate division, 600 million years ago. Apart from the coiled-coil domain, both TACC2 isoforms show isolated regions of homology with the other TACC proteins. Significantly, TACC2 contains two copies of the SDP repeat, which we recently identified as a binding site for the GAS41 protein [10]. Co-immunoprecipitation and in vitro analysis confirm that TACC2 also binds directly to this protein. GAS41 was originally believed to be a transcription factor [11,12], and, more recently, has been shown to interact directly with the INI-1 core component of the SWI/SNF chromatin remodeling complex [13]. We have now demon-

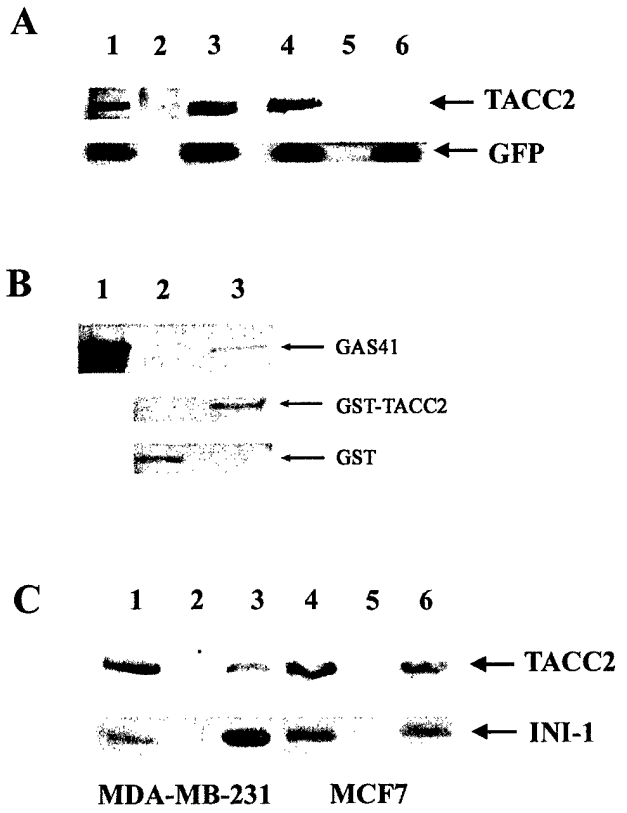

Fig. 5. Interaction between TACC2 and the SWI/SNF complex. (A) In vivo interaction of GAS41 with native TACC2s. Stable HEK293 transfectants expressing either GFP fused to GAS41 (EGGASB1) or GFP alone (EGFP/HEK293) were immunoprecipitated with either anti- GFP or rabbit IgG and immunoblotted with anti-TACC2 antibody. EGGASB1 and EGFP/HEK293 cells express the 120 kDa TACC2s isoform (lane 1 and 4) and this protein is specifically immunoprecipitated from EGGASB1 cells by the anti-GFP antibody (lane 3), but not control IgG (lane 2). No interaction between GFP and TACC2 was detected in immunoprecipitates of EGFP/HEK293 expressing GFP alone (lane 6). Lane 5 represents EGFP/HEK293 immunoprecipitated with control IgG. Bottom panels confirm that similar amounts of GFP-GAS41 (53 kDa) and GFP (27 kDa) are immunoprecipitated by the anti-GFP antibody in the lanes 3 and 6. (B). In vitro interaction of TACC2s and GAS41. Lane 1, 10% input of in vitro translated GAS41 protein; lane 2, GST as negative control; and Lane 3, GST-TACC2. Top panel: Autoradiograph of 8% SDS polyacrylamide gel with in vitro translated GAS41 protein pulled down with GST-TACC2. Bottom two panels represent Coomassie blue- stained gel of pull-down experiment demonstrating loading of GST fusion proteins. (C). Immunoprecipitation of native TACC2 by native INI-1 from breast cancer cell lines. TACC2 is expressed as the 120 kDa short isoform in MDA-MB-231 (Lane 1) and MCF7 (Lane 4). TACC2s is specifically immunoprecipitated by the anti-BAF47 (INI-1) antibody from both cell lines (Lanes 3 and 6), but not IgG (Lanes 2 and 5).

strated that TACC2 not only binds to the GAS41 protein, but can also be found in a complex with the SWI/SNF core subunit INI-1. A role for TACC proteins in transcriptional and post-transcriptional events has been shown for murine Tacc3 [3], and the Xenopus maskin protein [8], as well as implied for TACC1 [10,14]. This combined data suggests further that TACC proteins perform a scaffolding, assembly, or coordination function in bringing elements of the chromatin remodeling, transcriptional, and post-transcriptional machinery together in the nucleus.

We have previously mapped *TACC2* to chromosome 10q26, close to *FGFR2* [1]. The entire *TACC2* gene spans 23 exons dispersed over approximately 255 kb. Surprisingly, the long and short TACC2 isoforms differ by the inclusion of an unusually large 5 kb exon in the larger transcript. Analysis of the publicly available mouse genome database suggests that a similar-sized exon is present in the *TACC2* gene on mouse chromosome 7 (Still and Liang *unpublished* manuscript). The inclusion of large exons in *TACC* gene sequences appears to have occurred during the evolution of the *TACC* gene family. This is particularly evident when the human and mouse TACC3 sequences are examined [2]. In this case, a 996 bp exon has been acquired after the divergence of the primate and rodent lineages [2]. The significance of these sequences remains to be elucidated; however, large exons, such as exon 11 of *BRCA1,* which includes about 61% of the coding sequence of the gene, do appear to be hotspots for inactivating mutations in cancer [15]. *TACC2* maps to a region of the genome that exhibits loss of heterozygosity and deletions in many tumors, including breast [16], glioblastoma [17–19], lung [20], respiratory tract [21], prostate [22], and endometrial cancer [23,24]. Clearly, an examination of this exon in tumors derived from tissue that express the long isoform of TACC2 might reveal that TACC2 mutations underlie some of these tumor types.

It has been proposed that TACC2 is a class II tumor suppressor in that changes in expression, as opposed to mutations, can be linked to breast tumor progression [5]. Although we have not detected major changes in TACC2 protein levels in breast cancer cell lines, or RNA levels in resected tumor samples, analysis of TACC2-overexpressing cell lines revealed effects on the ability of breast cancer cells to exhibit anchorage independent growth and to migrate through a basement membrane-like matrix. TACC2s had little effect on the growth characteristics of the estrogen receptor positive MCF7 cell line, but significantly reduced the ability of estrogen receptor negative cell line MDA-MB-468 to grow in soft agar and to migrate through an extracellular matrix. Interestingly, the HMT-3522 cell line model, in which a potential role of TACC2 in breast tumorigenesis was first examined [5], also lack estrogen receptors [25], which could suggest that the effect of overexpression of TACC2s on the ability of breast cancer cells to divide in culture may be differentially affected by the genetic background of the original tumor.

In summary, we report the cloning and expression of the second member of the TACC gene family. The major 4.2 kb TACC2 transcript, which is expressed in the mammary gland, encodes a protein of 120 kDa. We failed to detect significant differential expression of this TACC2 isoform in cell lines and resected tumors, compared to normal controls. Therefore, additional immunohistochemical analysis of surgically resected breast tumors will need to be conducted to

define more accurately a link between TACC2 and breast tumorigenesis. We have also demonstrated that TACC2 interacts with GAS41 and INI-1, which suggests that TACC2 could play a role in transcription through interaction with the SWI/SNF complex. A proposed role of TACC2 in transcriptional events may explain how a decrease in TACC2 expression could be associated with the progression to a more malignant phenotype in the HMT-3522 cell line model [5].

Materials and methods

Expression analysis and cloning of the TACC2 cDNA

The TACC2 cDNA clone, FL4, was originally isolated from a fetal lung cDNA library during isolation of the TACC1 cDNA [1]. A subclone of this cDNA, lacking the conserved coiled-coil sequence, was hybridized to commercial multiple tissue Northern blots (BD Biosciences Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions. Additional cDNAs were identified by screening a human fetal brain cDNA library (BD Biosciences Clontech), according to standard protocols. cDNAs were then subcloned into pBSIISK(+) and sequenced. Primers designed to Genomescan predictions were: T2F77: 5′-GATTTCTCAAGTCACCCGCTTGGTC-3′, T2F340: 5′-ACATGGGCAATGAGAACAGCACC-3′, 1330F: 5′-GGAATTCCTCAGGGCCTG-3′, 2330F: 5′-GGGCCCCACTCTCTCAGACAG-3′, 2380R: 5′-GTCCTC-CTCACCCAGCTTG-3′, 3260F: 5′-AGCATGCGGTGATG-GTCAGTC-3′, 3260R: 5′-CCGCATGCTCCCTCTGGC-3′, 155R: 5′-GGTGGCTGTGTGCTGACCTC-3′ T2R6558: 5′-CTTTGCCCTCCTGATTCTTAGCGTC-3′. As previously described in [26], rt-PCR reactions were performed from commercially available RNA from colon, prostate, and mammary gland tissue (BD Biosciences Clontech). PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA) and sequenced by the Bioploymer Core Facility, Roswell Park Cancer Institute. The cDNA sequences of human long and short TACC2 isoforms have been deposited in the GenBank database with the Accession Numbers AF528099 and AF528098.

Immunologicals, cell lines, and Western blot analysis

Commercial antibodies were obtained from the following companies, and used according to manufacturers' instructions: anti-TACC2 rabbit polyclonal IgG (Upstate Biotechnology, Lake Placid NY, USA), anti-BAF47 (INI-1) (BD Transduction laboratories, Lexington, KY, USA), and anti-β-tubulin monoclonal antibody (Sigma Aldrich USA). Human embryonic kidney HEK293 cells and breast carcinoma cell lines MCF10A, T47D, HCC1395, HCC70, MCF7, MDA-MB- 231, MDA-MB-435, MDA-MB-468 and SK-Br3 cells were purchased from ATCC. MCF10A was cultured in chemically defined media [27]. The remaining cell lines were cultured in DMEM containing 10% fetal calf serum (RPCI Tissue culture media facility). Preparation of cell lysates, immunoprecipitation, and Western blot analysis were performed as previously reported [10].

In vitro analysis of TACC2 on tumor phenotype

The TACC2 short isoform was fused in frame to the green fluorescent protein (EGFP) in the EGFPC2 vector (BD Biosciences Clontech). This plasmid was transfected into MCF7 and MDA- MB-468, and stable transfectants selected as previously described [1]. Expression of the fusion protein was verified by fluorescence and Western blot analysis, and three stable transfectants from each cell line were then used for further analysis. In vitro analysis of transfected cell lines was carried out as previously described by Chen and colleagues [5]. The CellTiter 96 Non- radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was used for cell proliferation studies, according to manufacturer's instructions. Cells were plated at 5×10^3 cells per well in 96 well plates, and the medium replaced every two days. Proliferation assays were carried out 2, 4, 6, and 8 days after initial plating. For soft agar assays, cells were seeded at 1×10^5 cells per well in 0.35% agar in 12 well plates. Colonies 50 μm or greater were scored as positive. Invasion assays were performed using the Matrigel Invasion Chamber assay (Becton Dickinson, Bedford, MA, USA), according to manufacturer's instructions. 1×10^5 cells in culture medium lacking serum were introduced to the upper side of the invasion chamber. For the purpose of this experiment, culture medium containing 10% serum was used as the chemoattractant. After 24 hours at 37°C, the membrane was removed and invading cells on the lower surface fixed and stained. Cells were visualized by microscopy and counted. For all assays, each data point was performed in triplicate and differences between cell lines analyzed using one way ANOVA followed by Dunnett's Multiple Comparison Post Test (Graphpad Prism Version 3.0, Graphpad Prism Software Inc.).

In vitro glutathione-S-transferase pull-down assay

The TACC2s cDNA was cloned into the GST fusion vector pGEX5X2 (Amersham Biosciences, Piscataway, NJ, USA). GST domain and GST-TACC2 proteins were expressed in *Escherichia coli* BL21(DE3)plys 'S' with 1 mM IPTG at 37°C shaker for 2 h. Cells (100 ml) were harvested and resuspended in 10 ml of 1×phosphate buffered saline (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, protease inhibitors: 1 mM AEBSF, 0.8 μM Aprotinin, 50 μM Bestatin, 15 μM E-64, 20 μM Leupeptin, 10 μM Pepstatin A). The cells were lysed by sonication and the lysate cleared by centrifugation at 7500 rpm at 4°C for 20 minutes. The cleared lysate was immobilized on glutathione sepharose beads. The GAS41 cDNA was cloned into

pcDNA3 (Invitrogen, Carlsbad, CA, USA) and protein synthesized by TNT quick coupled transcription/translation system kit (Promega) and radiolabeled with ^{35}S methionine, according to manufacturer's instructions. About 5 μg of immobilized GST protein was incubated with 20 μl (40%) of the in vitro translated GAS41 protein in 750 μl of binding buffer (20 mM Tris-HCl, pH8.0, 0.2% Triton X-100, 2 mM EDTA pH8.0, 300 mM NaCl, and protease inhibitors) at 4°C for 90 min. Unbound GAS41 was removed from the sepharose beads by washing twice with binding buffer. The beads were then washed twice with wash buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA, 400 mM NaCl, protease inhibitors) that contained 0.2% NP-40. Bound proteins were eluted from the beads by boiling in an elution buffer (100 mM Tris-HCl, pH8.0, 20 mM reduced glutathione). The proteins were analyzed on 8% SDS polyacrylamide gels. Coomassie blue staining verified equal loading of the GST fusion proteins. Dried gels were autoradiographed.

Acknowledgments

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References

[1] I.H. Still, M. Hamilton, P. Vince, A. Wolfman, J.K. Cowell, Cloning of TACC1, an embryonically expressed, potentially transforming coiled-coil-containing gene, from the 8p11 breast cancer amplicon, Oncogene 18 (1999) 4032–4038.

[2] I.H. Still, P. Vince, J.K. Cowell, The third member of the transforming acidic coiled-coil-containing gene family, TACC3, maps in 4p16, close to translocation breakpoints in multiple myeloma, and is upregulated in various cancer cell lines, Genomics 58 (1999) 165–170.

[3] C.M. Sadek, et al., Isolation and characterization of AINT: a novel ARNT-interacting protein expressed during murine embryonic development, Mech. Dev. 97 (2000) 13–26.

[4] R.P. Piekorz, et al., The centrosomal protein TACC3 is essential for hematopoietic stem cell function and genetically interfaces with p53-regulated apoptosis, EMBO.J. 21 (2002) 653–664.

[5] H.M. Chen, et al., AZU-1: A Candidate Breast Tumor Suppressor and Biomarker for Tumor Progression, Mol. Biol. Cell 11 (2000) 1357–1367.

[6] J.J. Pu, C. Li, M. Rodriguez, D. Banerjee, Cloning and structural characterization of ECTACC, a new member of the transforming acidic coiled-coil (*TACC*) gene family: cDNA sequence and expression analysis in human microvascular endothelial cells, Cytokine 13 (2001) 129–137.

[7] F. Gergely, et al., The TACC domain identifies a family of centrosomal proteins that can interact with microtubules, Proc. Natl. Acad. Sci. USA 97 (2000) 14352–14357.

[8] B. Stebbins-Boaz, Q. Cao, C.H. de Moor, R. Mendez, J.D. Richter, Maskin is a CPEB-associated factor that transiently interacts with eIF-4E, Mol. Cell 4 (1999) 1017–1027.

[9] F. Gergely, D. Kidd, K. Jeffers, J.G. Wakefield, J.W. Raff, D-TACC: a novel centrosomal protein required for normal spindle function in the early Drosophila embryo, EMBO J. 19 (2000) 241–252.

[10] B. Lauffart, S.J. Howell, J.E. Tasch, J.K. Cowell, I.H. Still, Interaction of the transforming acidic coiled-coil 1 (TACC1) protein with ch-TOG and GAS41/NuBII suggests multiple TACC1-containing protein complexes in human cells, Biochem. J. 363 (2002) 195–200.

[11] U. Fischer, et al., Cloning of a novel transcription factor-like gene amplified in human glioma including astrocytoma grade I, Hum. Mol. Genet. 6 (1997) 1817–1822.

[12] J. Harborth, K. Weber, M. Osborn, GAS41, a highly conserved protein in eukaryotic nuclei, binds to NuMA, J. Biol. Chem. 275 (2000) 31979–31985.

[13] S. Debernardi, et al., The MLL fusion partner AF10 binds GAS41, a protein that interacts with the human SWI/SNF complex, Blood 99 (2002) 275–281.

[14] N. Conte, et al., Carcinogenesis and translational controls: TACC1 is down-regulated in human cancers and associates with mRNA regulators, Oncogene 21 (2002) 5619–5630.

[15] K. Grade, B. Jandrig, S. Schemeck, BRCA1 mutation update and analysis, J. Cancer Res. Clin. Oncol. 122 (1996) 702–706.

[16] F. Richard, et al., Patterns of chromosomal imbalances in invasive breast cancer, Int. J. Cancer 89 (2000) 305–310.

[17] K. Ichimura, E.E. Schmidt, A. Miyakawa, H.M. Goike, V.P. Collins, Distinct patterns of deletion on 10p and 10q suggest involvement of multiple tumor suppressor genes in the development of astrocytic gliomas of different malignancy grades, Genes Chromosomes Cancer 22 (1998) 9–15.

[18] L. Girard, S. Zochbauer-Muller, A.K. Virmani, A.F. Gazdar, J.D. Minna, Genome-wide allelotyping of lung cancer identifies new regions of allelic loss, differences between small cell lung cancer and non-small cell lung cancer as well as loci clustering, Cancer Res. 60 (2000) 4894–4906.

[19] P.A. Steck, et al., Functional and molecular analyses of 10q deletions in human gliomas, Genes Chromosomes. Cancer 24 (1999) 135–143.

[20] S. Petersen, et al., Chromosomal alterations in the clonal evolution to the metastatic stage of squamous cell carcinomas of the lung, Br. J. Cancer 82 (2000) 65–73.

[21] S. Petersen, et al., Analysis of the DMBT1 gene in carcinomas of the respiratory tract, Int. J. Cancer 88 (2000) 71–76.

[22] B. Leube, et al., Refined mapping of allele loss at chromosome 10q23–26 in prostate cancer, Prostate 50 (2002) 135–144.

[23] S. Nagase, et al., Deletion mapping on chromosome 10q25–q26 in human endometrial cancer, Br. J. Cancer 74 (1996) 1979–1983.

[24] S.L. Peiffer, et al., Allelic loss of sequences from the long arm of chromosome 10 and replication errors in endometrial cancers, Cancer Res. 55 (1995) 1922–1926.

[25] P. Briand, O.W. Petersen, B. Van Deurs, A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in a chemically defined medium, In Vitro Cell Dev. Biol. 23 (1987) 181–188.

[26] I.H. Still, J.K. Cowell, The t[8,13] atypical myeloproliferative disorder: further analysis of the ZNF198 gene and lack of evidence for multiple genes disrupted on chromosome 13, Blood 92 (1998) 1456–1458.

[27] H.D. Soule, et al., Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10, Cancer Res. 50 (1990) 6075–6086.